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STUDIES ON THE ULTRAFILTRATION AND ELECTRODIALYSIS OF INSULIN SOLUTIONS

DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
the Faculty of Pure Science of
Columbia University.

BY

CHARLES ERNEST BRAUN, B.S., A.M.

NEW YORK CITY
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TO
MY MOTHER AND FATHER

Acknowledgment

The author wishes to express his gratitude to Professor T. Clinton Taylor, at whose suggestion this investigation was undertaken, and under whose guidance it was carried out, for his kindly interest and helpful assistance.

The author also wishes to thank Professor Ernest L. Scott for his hearty co-operation in performing all the physiological tests recorded in this research.

STUDIES ON THE ULTRAFILTRATION AND ELECTRODIALYSIS OF INSULIN SOLUTIONS

INTRODUCTION

Material extracted from the pancreas[1], and subsequently subjected to more or less purification[2, 3], has gained increasing prominence as a therapeutic agent in the treatment of disturbed carbohydrate metabolism.

It was soon recognized, that these extracts contained substances other than the active principle, in quantities sufficient to completely obscure the properties of the active constituent, which has been called "Insulin." Although these solutions serve well enough for clinical uses, they are valueless for chemical investigations.

A considerable amount of experimental work has been done recently on insulin, both from the clinical and chemical viewpoint. The efforts bearing on the latter, have had for their purpose, mainly the concentration and isolation of the active constituent of insulin, although some work has been done on the chemical properties of the substance. The latter obviously, are but composite properties of a complex mixture of active material and accompanying impurities.

Briefly, the methods used for the purposes mentioned above, may be divided into two classes; fractional precipitation of the protein-like substances by regulation of the hydrogen ion concentration, and precipitation by various reagents. Among the first type may be noted the methods of Doisy, Somogyi and Shaffer[4, 5], and among the second, those of Collip[6], Dudley[7], Dudley and Starling[8], Moloney and Findlay[9], Best and Scott[10], Shonle and Waldo[11], and N. B. Fischer[12]. The only departure from either of the above methods is the meager and rather incomplete work of Locks and Hirsch[13, 14] on electrodialysis.

The purpose of this investigation was to find a method for separating or concentrating the active constituent in insulin solutions, so that the individual could be studied apart from the accompanying non-active materials.

Successful application of ultrafiltration through graded membranes, and of methods of electrophoresis and electrodialysis to the separation of amyloses in starch were lately carried on in this laboratory (Taylor and Iddles. Dissertation, Columbia 1925). These methods suggested themselves in attempting to purify insulin solutions.

Although the use of the first method did not give the desired result in the way of purification, nevertheless it did bring out some interesting properties of insulin. For this reason the technique has been given in some detail. On the other hand, the second method lent itself nicely to concentration of the physiologically active substance.

EXPERIMENTAL WORK

A. The Ultrafiltration of Insulin Solutions through Collodion Membranes of Varying Permeability.

The insulin* solutions used were the clinical U-20 Iletin (Insulin-Lilly) [15]. An apparatus, employing membranes, made and graded according to Nelson and Morgan[16], served to give all the data herein recorded. The plates, on which the membranes were made, were floated on mercury to insure perfect leveling, the only departure from the Nelson and Morgan technique.

The ultrafiltration unit consisted of the following parts, shown assembled and correspondingly lettered in Figure No. 1.

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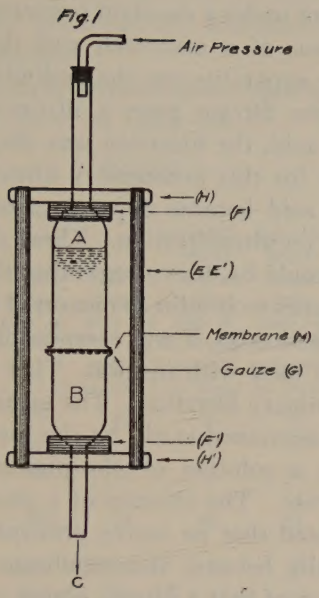
* The author wishes to express his thanks to Dr. G. H. A. Clowes, through whose courtesy the Eli Lilly Company supplied insulin for this investigation.



(A) Gooch funnel serving as an insulin compartment.

(B) Gooch funnel serving as the lower half of the ultrafilter.

(C) A receiving vessel for the filtrate.

(M) A permeable membrane.

(G) A nichrome gauze to support the membrane and prevent its rupture during filtration.

(EE′) Elastic bands to hold the upper and lower funnels rigid.

(HH′) Wooden collars, fitted on the funnels, over which the bands (EE′) were stretched.

(FF′) Felt buffers to prevent fracture of the glass due to the pressure exerted by the bands (EE′).

A tight seal between the upper and lower funnels, and the membrane was effected by means of a rubber-paraffin cement.

When the apparatus had been tested and found to be leakproof, an insulin solution, usually 15 cubic centimeters was placed in the upper part A, and a receiving vessel was placed in position C. Air, freed from carbon dioxide by passage through

soda lime, and kept under a constant pressure of 60 centimeters of water by means of a manostat, was then applied to the upper part of the apparatus and the preliminary filtration was begun. When the filtrate gave a distinct precipitate with phosphotungstic acid, the filtration was stopped.

The necessity for this preliminary filtration and test with phosphotungstic acid became apparent from some introductory experiments on ultrafiltration. These showed that before any conclusions could be drawn regarding the permeability of collodion membranes to insulin as measured by the physiological activity of the filtrates, it was essential first to saturate the membranes to be used with insulin. This was accomplished best by the preliminary filtration. The saturation of the membrane could be determined easily by the formation of a white precipitate when a solution of phosphotungstic acid[17] was added to the filtrate. The absence of a phosphotungstic acid precipitate indicated that no active principle had passed into the filtrate, usually because the membrane had not become saturated. The proof that a filtrate giving no precipitate with phosphotungstic acid is physiologically inactive is shown by the following experiment:

Five cubic centimeters of clinical U-20 insulin were ultrafiltered through a grade 15.7 membrane, which had not been saturated previously by a preliminary filtration. The filtrate gave no precipitate with phosphotungstic acid while the control sample gave a copious one under the same treatment. Samples of filtrate and control were tested biologically for blood sugar reducing properties.

The physiological activity in these samples and in all samples mentioned in subsequent work was tested out on rabbits weighing about 2 kilos. The standard injection per animal per kilo of body weight was one physiological insulin unit, which has been defined by the Toronto Insulin Committee[18] as, "one third of the amount required to lower the blood sugar below 0.045% and cause convulsions in a rabbit weighing 2 kilograms, which has been previously starved for 24 hours, within 2 to 5 hours after injection." Three samples of blood were drawn from each animal, an initial sample, one ¾ hours

after injection, and one 1¾ hours after injection. The sugar in each sample was quantitatively determined by the Shaffer and Hartmann[19] method.

The results of these physiological tests on the filtrate and control sample are given in Table No. 1.

TABLE 1.

Grade of Membrane used	Description of Material	A Initial Blood Sugar in mg. per 100 cc. blood	B Blood Sugar after ¾ hr. in mg. per 100 cc. blood	C Blood Sugar after 1¾ hr. in mg. per 100 cc. blood	D (A—B) Blood Sugar drop after ¾ hr. in mg. per 100 cc. blood	E (A—C) Blood Sugar drop after 1¾ hr. in mg. per 100 cc. blood	Remarks
15.7	Control	135	74	80	61	55	Clinical U-20 Insulin
15.7	"	141	66	68	75	73	gave good precipitate
15.7	"	130	68	74	62	56	with phosphotungstic acid
		135	69	74	66	61	Average
15.7	Filtrate	110	108	110	2	0	Gave no precipitate
15.7	"	122	118	118	4	4	with phosphotungstic
15.7	"	124	128	128	0	0	acid
15.7	"	122	128	122	0	0	
15.7	"	108	128	128	0	0	
15.7	"	105	118	114	0	0	
		115	121	120	0	0	Average

From the above data, it can be seen readily that the filtrate which gave no precipitate with phosphotungstic acid, was totally inactive, while the control, which gave a copious precipitate was active.

I. Effect of Ultrafiltration on Physiological Activity.

In all subsequent experiments, the membranes were saturated with insulin by a preliminary filtration, and then washed three times with 10 cc. of distilled water. A fresh insulin solution then was placed in part A, a clean receiving vessed at C, and the regular filtration was begun.

When about one half of the insulin solution had passed through the membrane, the filtration was stopped. Samples

of control, filtrate, and remainder on the membrane, were tested for physiological activity as described above.

This experiment was repeated for several membranes of varying grades of permeability with the hope of obtaining some fractionation of the active from the inactive material, as would be evidenced by increased activity of the solution above or below the membrane. Table No. 2 gives the results obtained.

TABLE 2.

Grade of Membrane used	Description of Material	A Initial Blood Sugar in mg. per 100 cc. blood	B Blood Sugar after ¾ hr. in mg. per 100 cc. blood	C Blood Sugar after 1¾ hr. in mg. per 100 cc. blood	D (A—B) Blood Sugar drop after ¾ hr. in mg. per 100 cc. blood	E (A—C) Blood Sugar drop after 1¾ hr. in mg. per 100 cc. blood	Remarks
20.7	Control	137	61	61	76	76	Clinical U-20 Insulin
12.0	"	122	63	73	59	49	1 Unit per Kilo was
11.5	"	135	69	71	66	64	injected
		131	64	68	67	63	Average
20.7	Filtrate	127	75	59	52	68	Filtrate from U-20
19.6	"	124	69	65	55	59	Insulin through mem-
12.0	"	121	68	64	53	57	branes whose grade
12.0	"	120	66	56	54	64	is given in first
11.5	"	134	64	62	70	72	column
3.6	"	134	71	53	63	81	
		127	69	60	58	67	Average
20.7	Remainder	110	64	57	46	53	Solution on mem-
19.6	"	136	74	62	62	74	brane when filtration
12.0	"	115	52	41	63	74	was stopped
11.5	"	123	59	56	64	67	
3.6	"	128	69	52	59	76	
		122	63	53	59	69	Average

Figures are averages for:

3 rabbits on samples: Control 20.7, 12.0, 11.5. Filtrate 20.7. Remainder 20.7.
4 " " " Filtrate 3.6. Remainder 3.6.
5 " " " Filtrate 12.0.
6 " " " Filtrate 19.6, 12.0, 11.5. Remainder 19.6, 12.0, 11.5.

From the above data, which represent averages of series of animal results on each sample tested, it is seen that within the experimental error, the average sugar drop per unit injected is the same for the control, filtrate and remaining solution. It may be concluded, then, that the active principle of insulin preparations can be ultrafiltered through collodion membranes of varying permeability without loss of physiological activity, and further, that no apparent fractionation takes place, during the ultrafiltration.

The fact that all of the active principle passed through the membrane is contrary to the observations of Best and MacLeod [20], Dudley[7], Murlin, Piper and Allen[21], and Shonle and Waldo[11]. The membranes used by these investigators, however, were probably much less permeable than those used in our experiments.

II. Nitrogen Changes During Ultrafiltration of Insulin Solutions.

The practicability of ultrafiltration of insulin solutions through graded membranes without loss of physiological activity having been shown, it was decided next to study the nitrogen content of the filtrate, with the view of ascertaining whether nitrogen could be removed from insulin by ultrafiltration without impairing its activity. This could be accomplished best, by collecting fractions of the filtrate and determining the nitrogen content and physiological activity in each.

The ultrafiltration apparatus and membrane technique used was the same as described in the previous section, with the exception, that each successive five cubic centimeters of filtrate were collected in separate vessels, and the membrane was not saturated previously. The nitrogen content in each fraction was then quantitatively determined by the micro-Kjeldahl technique of Pregl [22], and each fraction was tested for precipitation with phosphotungstic acid. This was done prior to testing the fraction for physiological activity, because as has been mentioned previously, the absence of a phosphotungstic acid precipitate indicates the absence of activity in the filtrate.

Several fractional ultrafiltration experiments were carried out with membranes of different degrees of permeability ranging from 2.0 to 12.0, (graded according to Nelson and Morgan[16]) and with insulin solutions on both the acid and alkaline sides of pH=4.8 (the recorded isoelectric point of Insulin[15]). The results obtained in all these investigations are shown by the detailed data from two experiments, both on the same grade membranes, but on different sides of the isoelectric point. Concurrently, a colorimetric determination of the hydrogen ion[23] concentration on each fraction was made. The data obtained has been recorded in Table No. 3 and is shown graphically in Figures No. 2 and No. 3.

TABLE 3—PART A.

Grade membrane used = 10.0
Initial pH = 3.7 (acid side of isoelectric point)

	A	B	C	D (A—B)	E (A—C)			
Description of Material	Initial Blood Sugar in mg. per 100 cc.	Blood Sugar after ¾ hr. in mg. per 100 cc.	Blood Sugar after 1¾ hr. in mg. per 100 cc.	Blood Sugar drop after ¾ hr. in mg. per 100 cc.	Blood Sugar drop after 1¾ hr. in mg. per 100 cc.	Nitrogen Content in mg. N_2 per cc.	pH of Filtrate Fraction	Remarks
Control	117	52	65	65	52	0.234	3.7	Clinical U-20 Insulin.
Filtrate Fraction No. 1						0.076	3.7	No ppt. with phospho-tungstic acid
Filtrate Fraction No. 2						0.151	4.0	Very slight ppt. with phospho-tungstic acid
Filtrate Fraction No. 3						0.225	4.1	Some ppt. with phospho-tungstic acid
Filtrate Fraction No. 4						0.230	4.2	
Filtrate Fraction No. 5						0.233	4.3	Heavy ppt. with phospho-tungstic acid

Filtrate Fraction No. 6	143	81	70	62	73	0.233	...	Volume too small for pH.
Filtrate Fraction No. 7						0.231	4.5	

TABLE 3—PART B.

Grade membrane used = 10.0
Initial pH = 6.1 (alkaline side of isoelectric point)

	A	B	C	D	E			
Description of Material	Initial Blood Sugar in mg. per 100 cc.	Blood Sugar after ¾ hr. in mg. per 100 cc.	Blood Sugar after 1¾ hr. in mg. per 100 cc.	(A—B) Blood Sugar drop after ¾ hr. in mg. per 100 cc.	(A—C) Blood Sugar drop after 1¾ hr. in mg. per 100 cc.	Nitrogen Content in mg. N_2 per cc.	pH of Filtrate Fraction	Remarks
Control	126	86	67	40	59	0.234	6.1	U-20 Insulin adjusted to pH 6.1
Filtrate Fraction No. 1						0.084	6.1	No ppt. with phospho-tungstic acid
Filtrate Fraction No. 2						0.146	6.1	Very slight ppt. with phospho-tungstic acid
Filtrate Fraction No. 3						0.218	6.1	Ppt. with phos-photungstic acid
Filtrate Fraction No. 4						0.233	6.1	
Fraction No. 5	122	64	62	58	60	0.235	6.1	Heavy ppt. with phospho-tungstic acid
Filtrate Fraction No. 6						0.231	6.1	
Filtrate Fraction No. 7	135	60	49	75	86	0.233	6.1	2 rabbits took convulsions

FIG. 2

NITROGEN CHANGES DURING ULTRAFILTRATION OF INSULIN SOLUTIONS

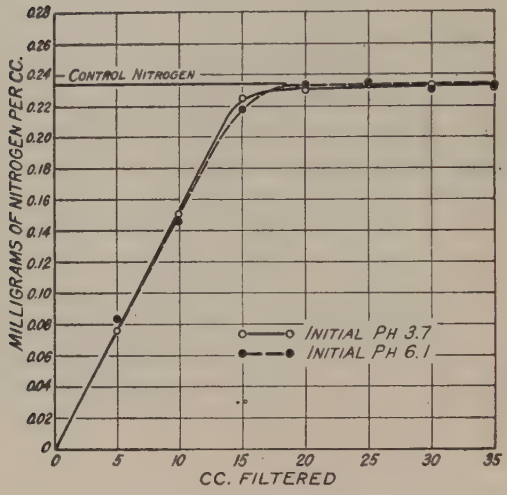
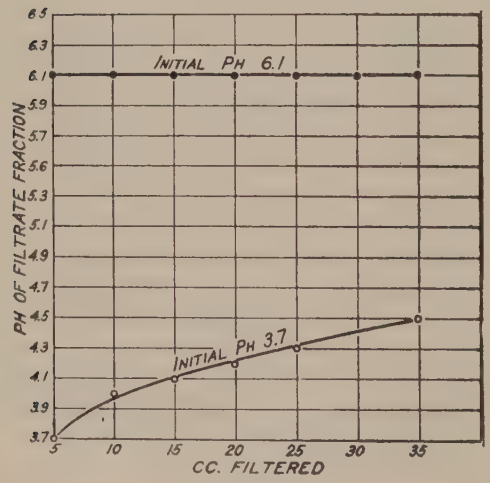


FIG. 3

PH CHANGES DURING ULTRAFILTRATION OF INSULIN SOLUTIONS



From the above results, it is apparent, that a low nitrogen content of the filtrate, probably caused by loss in saturating the membrane, is accompanied by an equivalent drop in physiological activity.

Since collodion membranes removed hydrogen ion from solutions[24, 25], it is to be expected, that the insulin solution above the membrane should become progressively more alkaline as the ultrafiltration continues. As soon as a pH of about 4.8 was reached, precipitation of insulin started. On the alkaline side of the isoelectric point, this change in hydrogen ion did not take place, and no experimental difficulty, caused by precipitation of insulin on the membrane during the ultrafiltration was experienced.

B. The Electrodialysis of Insulin Solutions.

I. The Formation of an Active Sediment by Electrodialysis of Insulin Solutions.

The inactivation of insulin by both oxidation and reduction[11], demanded the use of a special type of electrolytic cell for the investigation of the behavior of the active principle when subjected to electrodialysis. The essential feature in the design of the cell must be therefore, complete removal of the electrodes from the insulin solution undergoing electrodialysis. This would help prevent oxidation or reduction of the active principle by any secondary products of the electrolysis, formed at the electrodes.

The Electrolytic Cell:

The cell finally adopted and used in all experiments on elec trodialysis, was composed of the following parts, shown assembled and correspondingly lettered in Figure No. 4.

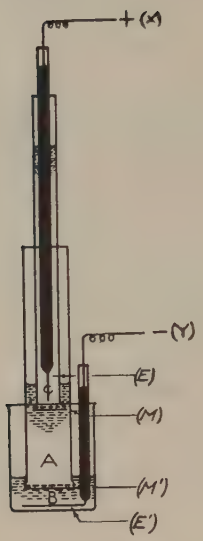
(A) An insulin compartment filled with about 25 cc. of U-20 insulin.

(BC) Electrode compartments filled with 20 cc. of distilled water.

(EE′) The platinum electrodes.

(MM′) Impermeable membranes.

Fig. 4



The insulin compartment (A) and electrode compartment (C) were sealed off by means of collodion membranes (MM'), made in such a manner as to render them impermeable to the active principle but permeable to electrolytes during the electrodialysis.

The Electrodialysis Membranes:

The membranes (MM') mentioned above, were prepared by the following method. The end of the cell unit upon which the membrane was to be made, was covered with a tightly stretched piece of cheese-cloth, (shown as dotted lines in Figure No. 4) which served as a framework for the membrane. The covered end of the unit was then dipped into a fairly pure collodion solution (Merk's Collodion), removed, and revolved for ten minutes. At the end of this time all of the ether and most of the alcohol had evaporated, leaving on the cloth gauze a rather impermeable membrane. This was repeated three times, thereby insuring against leaks, and giving a very impermeable membrane. These were soaked then, in distilled

water for at least five hours before being used. They did not "blush" in contact with water but remained perfectly transparent.

The Electrodialysis Technique:

When the cell had been set up as shown in Figure No. 4, the insulin solution to be electrodialysed was placed in compartment (A), the proper volume (20 cc.) of distilled water in compartments (B) and (C), and the current turned on. A potential of 100 volts was applied between (x) and (y) by means of direct current from light service, passing through suitable resistance. With this drop in potential across (x) and (y), a current of 5 milliamperes flowed through the cell. In all subsequent experiments, the polarity of the cell, unless otherwise stated, was that shown in Figure No. 4, with (C) the anode and (B) the cathode compartment.

Current was passed for any desired period of time. The circuit was then broken, compartment (A) was disengaged from the cell, and the liquid decanted from the sediment which had been formed on the membrane (M') during the electrodialysis.

The sediment was washed into a small Büchner funnel by means of absolute methyl alcohol. The residue on the filter was washed several times with absolute alcohol and finally with absolute ether. This last treatment is not absolutely essential, but it produces an almost white sediment instead of a dark brown one and materially increases the ease with which the substance can be removed from the paper. The sediment was removed from the filter and dried in vaccuo at 30° C. to constant weight.

All subsequent references to dry weight are based on constant weight at 30° C. in vaccuo. It is impossible to dry at higher temperatures, since insulin rapidly becomes inactivated if heated above 30° C. [20, 4, 7, 21].

II. The Physiological Activity of the Sediment.

The physiological activity of the sediment produced by the electrodialysis of insulin solutions was tested, in order to de-

termine the effect of the electric current on the potency of the active principle.

The dried sediment was weighed out in 2 to 2.5 milligram samples, and dissolved in such a volume of physiological salt solution (0.9% sodium chloride solution) acidified with 4 drops of N/10 hydrochloric acid per 2 cc. of solution, as would give a concentration of 1 milligram per cc. Then ½ cc. of this solution was diluted to 10cc. with physiological salt solution, giving a final concentration of 0.05 milligrams per cc. of solution. The standard injection was 1 cc. of this diluted solution or 0.05 milligrams per kilo of body weight. Control samples were made up in exactly the same manner to contain about 0.1 milligrams per cc. of final solution, and were injected at about 0.1 milligrams per kilo of body weight. The animal technique and blood sugar determinations were carried out exactly as described under ultrafiltration. Several samples of sediment prepared from different batches of insulin, together with adequate controls, were tested for physiological activity as described, and the results obtained have been recorded in Table No. 4.

TABLE 4.

Exp. No.	Description of Material	Mg. per kilo injected	A Initial Blood Sugar in mg. per 100 cc. blood	B Blood Sugar after ¾ hr. in mg. per 100 cc. blood	C Blood Sugar after 1¾ hr. in mg. per 100 cc. blood	D (A—B) Blood Sugar drop ¾ hr. in mg. per 100 cc. blood	E (A—C) Blood Sugar drop 1¾ hr. in mg. per 100 cc. blood	Remarks
1 & 2	Control	0.12	120	58	59	62	61	Clinical U-20 Iletin
3	"	0.10	129	81	63	48	66	(Insulin-Lilly)
4 & 5	"	0.13	124	65	60	59	64	
7 & 8	"	0.10	134	74	57	60	77	
9 & 10	"	0.12	129	77	77	52	52	
15	"	0.10	135	67	58	68	77	
12, 13, 16	"	0.10	144	67	71	77	73	Concentrated non-clinical Iletin.
			131	70	65	61	67	Average

1	Sediment	0.07	132	120	119	12	13	Insulin in contact with electrodes.
3	"	0.05	125	57	61	68	64	First active sample
4	"	0.05	127	56	42	71	85	
5	"	0.05	150	110	96	40	54	Ppted from (CH_3OH—HCl) by dry ether.
7	"	0.05	142	74	63	68	79	Formed at pH 3.6—
8	"	0.05	121	60	63	61	58	Formed in ½ hr. at
9	"	0.05	135	74	69	61	66	pH 3.6—3.7. From
10	"	0.05	144	58	77	86	67	clinical U-20 Iletin.
15	"	0.05	133	79	68	54	65	From clinical U-20 Iletin.
12	"	0.05	127	72	77	55	50	From conc. non-
13	"	0.05	127	70	69	57	58	clinical Iletin.
16	"	0.05	149	71	63	78	86	
			133	67	65	66	68	Averages on Nos. 3, 4, 7, 8, 9, 10, 15, 12, 13, 16.

NOTE: Figures above are averages on data from 3 to4 rabbits per sample tested.

From the data given in Table No. 4, it can be seen that the average blood sugar drop for ¾ hours and 1¾ hours after injection, on 7 control samples, was 61 and 67, while the average on 10 comparable sediment samples for the same periods of time, was 66 and 68 milligrams per 100 cc. of blood, or that the average activities were about equal. But from Table No. 4, it also will be noted, that the unit-weight of solid material injected per kilo, was not equal for the control and sediment samples, the latter being only 0.05 milligrams as compared to 0.1 milligrams of the former, or one half as much. Since half the unit weight of sediment produces about the same lowering of blood sugar in the experimental animals, as a whole unit weight of original insulin, it has been concluded that the sediment produced by the electrodialysis of insulin solutions is apparently twice as active, physiologically, as the original material. As will be shown later, these results take into consideration the experimental errors.

III. The Physiological Activity of the Filtrate.

The greater activity of the sediment could be explained best by assuming that some relatively inactive substance was

being removed from the active material during the electrodialysis of insulin solutions, or in other words, the original material was being fractionated in some way into two substances, one of high activity, and one of low activity. The validity of this assumption can be shown by the isolation of a relatively inactive product from the filtrate remaining after the removal of the active sediment.

This was attempted, and an organic substance showing little physiological activity was obtained from the filtrate by two different procedures. These consisted of (a) evaporation of the clear filtrate from the electrodialysis to constant weight at 30° C. in vaccuo, and (b) adjustment of the pH to 4.8, filtration of the precipitate formed, and drying the latter in vaccuo as usual. Method (a) gave a substance which showed greater activity than the material produced by (b) as can be seen from the physiological results on filtrate samples recorded in Table No. 5.

TABLE 5.

Exp. No.	Description of Material	Mg. per kilo injected	A Initial Blood Sugar in mg. per 100 cc. blood	B Blood Sugar after ¾ hr. in mg. per 100 cc. blood	C Blood Sugar after 1¾ hr. in mg. per 100 cc. blood	D (A—B) Blood Sugar drop ¾ hr. in mg. per 100 cc. blood	E (A—C) Blood Sugar drop 1¾ hr. in mg. per 100 cc. blood	Remarks
3	Filtrate	0.05	111	87	100	27	11	Produced by evap-
4	"	0.05	125	93	104	32	21	oration to constant
15	"	0.05	127	82	111	45	16	weight at 30° C.
16	"	0.05	135	103	104	32	31	
12	"	0.05	139	129	132	10	7	Produced by ad-
16′	"	0.05	126	103	122	23	4	justment of pH.
			127	99	112	28	15	Average

The average activity for the six filtrates was 28 and 15, as compared to 66 and 68 milligrams of sugar per 100 cc. of blood, for the same time intervals after injection, thereby showing, that the physiological activity of the organic substance remaining in the filtrate after removal of the active

material is less than half that of the sediment. Even though these figures do not represent an absolute cleavage of the original insulin into an active and inactive substance, which would be a very difficult accomplishment as shown by some of the work presented in following sections, nevertheless, they do indicate that the production by electrodialysis of a substance of increased activity is due to fractionation of the original material.

IV. The Deposition of the Active Sediment.

The deposition of an active sediment during the electrodialysis of insulin solutions had been established previously, but no change either of the polarity of the cell or of the initial pH of the solution had been attempted. It was of interest to investigate both of these changes from the point of view of determining whether the active material always deposited at the same electrode, or migrated in different directions, depending on the hydrogen ion concentration of the solution.

The usual electrodialysis was carried out with the polarity of the cell arranged so that the lower membrane was the cathode, and with the pH of the solution about 3.7, the acid side of the isoelectric point. As has been stated, under these conditions, the sediment was always deposited at the lower or cathodic membrane. Under such experimental conditions, the cathodic deposition of the active material left two points open for discussion, namely, was the deposition on the cathodic membrane due to distinct cathode migration, or was it due to the material being drawn down to the lower membrane, which happened to be the cathode, merely by the force of gravitation.

In order to settle these points, experiments were performed with the initial pH of the solution at 3.7, but with the cell polarity reversed, thus making the lower membrane the anode, and the upper one the cathode. Under these conditions, the active material always migrated to the upper membrane against the force of gravity showing that when insulin solutions on the acid side of the isoelectric point were subjected to electrodialysis the cathodic deposition of active sediment was

not due to gravitation, but was caused by a distinct cathodic migration of material carrying the activity.

The next point to be investigated was obviously, the effect of changing the pH of the insulin solution from the acid to the alkaline side of the isoelectric point on the direction of migration of the active material. Experiments were performed with the pH of the insulin solutions adjusted to about 9.0 and kept on the alkaline side of the isoelectric point (pH=4.8) by the continuous addition of dilute caustic soda solution. In all the experiments the deposition took place at the anodic, instead of at the cathodic membrane. Thus it has been shown that the deposition of an active sediment during electrodialysis of insulin solutions is caused by a definite migration of a material carrying the activity, and that the direction of migration is dependent upon the hydrogen ion concentration of the solution undergoing electrodialysis.

V. Deposition of the Active Sediment at a Definite pH.

In preliminary experiments on the electrodialysis of insulin solutions, it had been observed that the pH of the solution changed as the electrodialysis progressed, as was to be expected, since the process combined dialysis with electrolysis. It was decided to study these pH changes more thoroughly with the idea of perhaps gaining more insight into the nature of the process which was causing the formation of the active sediment.

It has been shown in the previous section that the deposition of a sediment during electrodialysis of insulin solutions occurs on both the acid and alkaline side of the isoelectric point (pH = 4.8). The experimental difficulties involved in maintaining an alkaline solution during the electrodialysis, and more important, the rapid inactivation of insulin in alkaline solutions[7, 11], made it impossible to study the pH changes on the alkaline side of the isoelectric point. Therefore, only the pH changes on the acid side have been investigated.

The technique employed for the study of the pH changes during electrodialysis was of a type which not only followed every change in pH, but also determined the constancy of pH

in different parts of the solution. This twofold result was accomplished by means of two capillary pipettes, one placed at the very bottom, and the other at the very top of the solution in the cell. By using a "Y" tube connection between the two capillary pipettes, it was possible to draw off liquid simultaneously from the very top and very bottom of the solution in the cell, thus minimizing the possibility of a change in pH by diffusion during the removal of the samples. The pH of the top and bottom samples and also of the mixed solution was determined at different time intervals over a period of 24 hours by the colorimetric procedure previously referred to. The results obtained in three experiments on clinical U-20 insulin have been averaged, and are recorded in part A of Table No. 6.

TABLE 6.

Part A.

Time	Appearance of Solution	Wgt. of Sediment Deposited	Determined pH of solution at top of cell	on mixed solution	at bottom of cell
start	clear		3.7	3.7	3.7
½ hr.	"	8.3 mg.	3.6	3.6	3.6
1 "	"	not weighable	3.6	3.6	3.6
1½ "	"		3.7	3.7	3.7
2 "	"		3.9	3.8	3.8
2½ "	turbid		4.7	4.7	4.7
3 "	"				
5 "	"		4.9	4.9	4.9
7½ "	"		4.9	4.9	4.9
10 "	"		5.0	5.0	5.0
24 "	clear		5.1	5.1	5.1

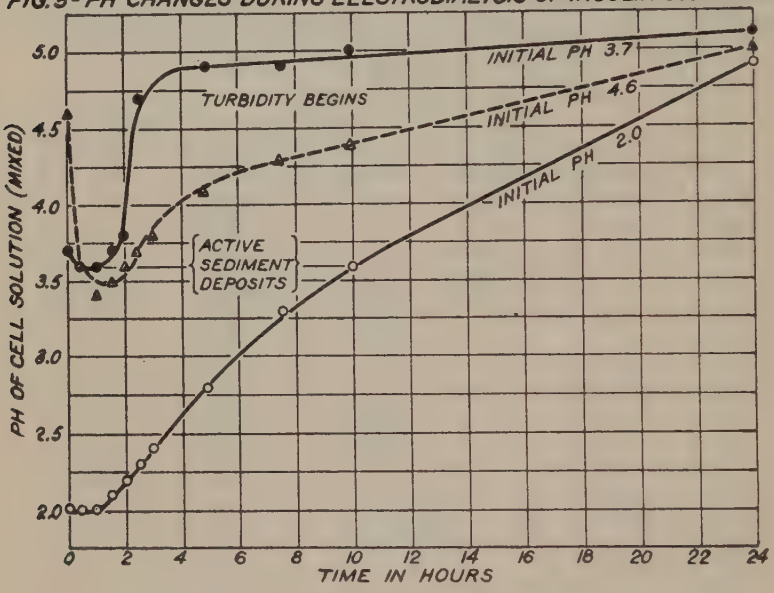
Part B.

start	clear		2.0
½ hr.	"		2.0
1 "	"		2.0
1½ "	"		2.1
2 "	"		2.2
2½ "	"		2.3
3 "	"		2.4
5 "	"		2.8
7½ "	"		3.3
10 "	"	7.3 mg.	3.6
24 "	turbid		4.9

Part C.

start	clear		4.6
½ hr.	"	7.2 mg.	3.6
1 "	"	trace	3.4
1½ "	"		3.5
2 "	"		3.6
2½ "	"		3.7
3 "	"		3.8
5 "	"		4.1
7½ "	"		4.3
10 "	"		4.4
24 "	turbid		4.9—5.0

FIG. 5 - PH CHANGES DURING ELECTRODIALYSIS OF INSULIN SOLUTIONS



From these results it can be seen that when an insulin solution with an initial pH of 3.7 is subjected to electrodialysis there is a distinct drop in pH during the first part of the process, followed by a progressive increase in pH, and further, that these pH changes are the same in all parts of the cell within the experimental error of the pH determinations. It is to be noted also, that accompanying the drop in pH of the solution is the deposition of all the weighable amount of

active sediment. Furthermore, turbidity, followed by the formation of a boundary which very gradually settles down to the lower membrane, begins at a pH of about 4.7—4.8, or within the isoelectric range of the original insulin solution.

In order to determine whether these particular pH changes were specific for insulin solutions with an initial pH of 3.7, experiments were performed on solutions having a starting pH other than 3.7. Since the experiments recorded in part A of Table No. 6 showed that the pH was constant throughout the entire cell, only the pH of the mixed solution was determined in the work which is about to be described.

Two experiments on clinical U-20 insulin with an initial pH of 2.0 and two on the same material but with an initial pH of 4.6, were performed, and the results obtained were averaged and recorded in Table No. 6 parts B and C respectively. The data given in Table No. 6 has been shown graphically in Figure No. 5.

When the initial pH of the insulin solution was 2.0 nothing visible occurred during the course of the electrolysis until the pH had become adjusted to about 3.6, at which point practically the entire amount of active sediment deposited on the cathodic membrane, as was the case when the initial pH was 3.7. As the process continued, the pH of the solution gradually increased until, at a pH of about 4.8 the solution became turbid, as had been observed in the previous experiments.

When clinical U-20 insulin adjusted to an initial pH of 4.6 was electrodialysed, a very pronounced change in pH occurred during the first part of the process. The pH of the solution dropped from 4.6 to about 3.4 and all the weighable sediment was deposited on the cathodic membrane at a pH of about 3.6. As the electrodialysis continued, the pH of the solution progressively increased, until at a pH of about 4.8, the usual turbidity set in.

These results have led to the conclusion that the deposition of an active sediment during electrodialysis of insulin solutions having an initial pH on the acid side of the isoelectric point (4.8), always takes place at a definite pH, which appears to be about 3.6. It has also been concluded, that the appear-

ance in the solution of a turbidity at a pH of about 4.8, followed by the formation of a boundary which gradually settles to the lower membrane, is due to an effect other than that which causes the deposition of the active sediment.

VI. Deposition of the Active Sediment is Not Due to Isoelectric Point Precipitation.

In the investigation of the pH changes in insulin solutions undergoing electrodialysis, it has been shown that the deposition of the active sediment takes place from a perfectly clear solution, when the pH has become adjusted to about 3.6. Furthermore, the clear solution becomes turbid at a pH of about 4.8. Since the isoelectric point of the original material is at a pH of about 4.8 [4, 15], and since the pH at which the deposition of the active sediment takes place is about 3.6, it appears, that the separation of the active material during electrodialysis of insulin solutions is not due to an isoelectric point precipitation.

The change in direction of migration of the sediment, as shown in section four, indicates that this material must be amphoteric. Until it can be demonstrated experimentally that the isoelectric point of the active sediment is at a pH other than 3.6, the argument advanced above is open to discussion because, if the isoelectric is at 3.6 then the deposition of active sediment might be due to insolubility of the material at this particular pH. Several experiments were performed to prove that this deposition of active material was not due to isoelectric point precipitation.

In the first place, the isoelectric point of the active sediment was experimentally determined. Samples of dried sediment were dissolved in distilled water acidified with dilute hydrochloric acid. Very dilute alkali ($^{N}/_{200}$) was then gradually added with constant shaking until a precipitate appeared. The pH at which maximum precipitation occurred was determined colorimetrically and the addition of alkali was then continued until the precipitate dissolved, showing the amphoteric nature of the substance. Other samples of the same sediment were dissolved in distilled water, made alkaline with dilute alkali. The same procedure, as just described, was carried out

excepting that dilute hydrochloric acid ($^{N}/_{100}$) was added instead of dilute alkali. The pH, at which the maximum precipitation occurred, or the isoelectric point of the active sediment, was found, as above described to be about 5.2. Since the pH at which deposition of this material takes place has been shown experimentally to be about 3.6, while the isoelectric point of the sediment has been found to be at a pH of about 5.2, it can be seen, that the deposition of active sediment during electrodialysis of insulin solutions is not due to an isoelectric point precipitation.

Other experimental proof for the validity of the above statement, is had in the fact that if the circuit was broken just after the material had been deposited at a pH of about 3.6, the sediment entirely redissolved in a short time. This was to be expected, since this same active sediment is completely soluble in water at a pH of about 3.6.

Furthermore, if the insulin solution undergoing electrodialysis was agitated rapidly by stirring, no deposition of active sediment occurred even after two hours. When the stirring was stopped, the usual deposit formed. If an isoelectric point precipitation were the cause of the deposition of an active sediment during electrodialysis of insulin solutions, agitation of the solution should have no effect of the formation of the precipitate.

Therefore, it has been demonstrated by three independent experiments that the deposition of the active sediment from insulin solutions subjcted to electrodialysis is not due to an isoelectric point precipitation.

The results of these experiments also show the difficulty of obtaining a filtrate with absolute physiological inactivity, due not only to the solubility of the active material at a pH of 3.6, but also to the overlapping of the isoelectric ranges (4.8—5.0 and 5.0—5.2) of the inactive and active constituents respectively.

The turbidity which has been shown to begin at a pH of about 4.8 and which is followed by the formation of a definite boundary and settling of the latter on the lower membrane, is probably due to isoelectric point precipitation of the inactive

component. [This pH (4.8—5.0) is the isoelectric point of insulin usually recorded in the literature [4, 5, 7, 15].] This was shown experimentally by the failure of the flocculated material to redissolve when the circuit was broken, and to almost immediate resolution upon the addition of either acid or alkali.

The relative inactivity of the material flocculated out of the solution of a pH of about 4.8, due probably to isoelectric point precipitation has been shown by a quantitative experiment. A solution of clinical U-20 insulin with an initial pH of 3.7, was divided into two equal volumes. Both portions were independently electrodialysed in the usual manner. The process was stopped and the sediment removed from one solution when the pH had become adjusted to about 3.6, and from the second at a pH of about 4.8. The solids from each were dried as usual and tested for physiological activity on the same weight basis. The results obtained are recorded in Table No. 7.

TABLE 7.

Description of Material	Mg. per kilo injected	A Initial Blood Sugar in mg. per 100 cc. blood	B Blood Sugar after ¾ hr. in mg. per 100 cc. blood	C Blood Sugar after 1¾ hr. in mg. per 100 cc. blood	D (A—B) Blood Sugar drop after ¾ hr. in mg. per 100 cc. blood	E (A—C) Blood Sugar drop after 1¾ hr. in mg. per 100 cc. blood	Remarks
Sediment	0.05	128	80	82	48	46	Formed at pH 4.8
"	0.05	116	77	84	39	32	
"	0.05	119	66	70	53	49	
		121	74	79	47	42	Average
Sediment	0.05	158	102	108	56	50	Formed at pH 3.6
"	0.05	138	46	18	92	120	
"	0.05	154	88	72	66	82	
"	0.05	119	60	56	59	63	
		142	74	63	68	79	Average

From these results it can be seen that the material flocculated at a pH of about 4.8 showed an average blood sugar drop of only 47 and 42, as compared to 68 and 79 for the sediment

deposited at pH 3.6. This experimentally indicated that the material which settles out of the solution at a pH of about 4.8, after the sediment has become deposited at a pH of about 3.6, contains little physiological activity. Contamination of the active sediment by this product materially decreases the potency of the active substance. This experimental evidence strongly indicates a fractionation of insulin by electrodialysis, as suggested in section three and shows also, that it is essential to stop the electrodialysis of insulin solutions before the pH has become adjusted to about 4.8, in order to prevent contamination and subsequent decrease in potency of the active material by the relatively inactive substance produced at this pH.

VII. Chemical Investigation of the Active Sediment.

In the previous sections it has been shown that an active sediment is formed during the electrodialysis of insulin solutions, from causes other than isoelectric precipitation. An investigation into the physical and chemical characteristics of the active substance has been undertaken with the hope of throwing some light on the chemical nature of the active principle.

The active deposit from insulin solutions subjected to electrodialysis is white when first prepared, but on exposure to the air, it gradually developed a grayish color. Under microscopic examination the active sediment shows sharp, ragged edges, like pieces of broken glass but no definite crystalline structure.

The active substance is soluble in dilute hydrochloric, sulfuric, and acetic acids, and in dilute caustic soda solutions. It is insoluble in absolute and 95% ethyl alcohol, absolute ether, acetone, carbon tetrachloride, benezene, petroleum ether, and ethyl acetate. It shows slight solubility in absolute methyl alcohol saturated with dry hydrogen chloride. The sediment when dissolved in acidified water, imparts a pale yellow color to the solution. In alkaline solution this yellow color is much more pronounced.

The active material is precipitated from acidified aqueous solutions by phosphotungstic acid. It can also be precipitated

from aqueous solutions by the addition of a large volume of absolute ethyl or methyl alcohol. If dry ether is added to a solution of the active substance in absolute methyl alcohol saturated with dry hydrogen chloride, a non-crystalline product is precipitated which shows slightly less than normal physiological activity as can be seen from experiment No. 5 recorded in Table No. 4. The average sugar drop for the material precipitated as described was 40 and 54, as compared to 59 and 64, in the same time intervals for the control sample.

The material burns readily, giving rise to an odor resembling burning hair and leaves a white fluffy ash. Quantitative determinations for the percentage ash in several samples of sediment have been recorded in Table No. 8. The ash determinations were made in a small platinum dish on samples ranging from 20 to 30 milligrams by ignition at red heat to constant weight. The ash is not alkaline, and its insolubility in dilute acids prompted a chemical investigation. This was done by the usual method [26, 27] with sulfuric acid and hydrofluoric acid, used in determining silica. The results obtained have been recorded in Table No. 8.

TABLE 8.

Sample No.	Description of Material	% Ash non-volatile after ignition	% Ash non-volatile after H_2SO_4–H_2F_2 treatment	% SiO_2 in Ash	% N_2 Based on total solids	% N_2 based on organic solids	Remarks
4	Control				14.15		Clinical U-20 Iletin
9	"				13.89		(Insulin-Lilly)
10	"				14.21		
15	"				13.51		
12, 13, 16	"	3.82	1.53	60.00	12.35	12.84	Conc. non-clinical Iletin
4	Sediment				18.68		Made from clinical
9	"				13.88		U-20 Iletin
10	"				13.66		(Insulin-Lilly)
15	"				13.62		
12	"	2.71	0.00	100.00	14.62	15.02	Made from conc.
13	"	2.50	0.00	100.00	14.12	14.48	non-clinical Iletin
16	"	1.30	0.00	100.00	13.87	14.06	
						14.52	Average

4	Filtrate	12.18	From U-20 clinical Iletin
12	"	13.78	From conc. non-
13	"	13.40	clinical insulin
16	"	12.11	

Each figure represents the average of 2 determinations.

It can be seen from the tabulated data, that the ash from three different samples of sediment consistd entirely of silica, while the ash from the control, from which these sediments had been produced, consisted of only 60 per cent. silica. Since the ash from the control samples showed a distinctly alkaline reaction toward phenolphthalein, while the ash from the sediments did not, it can be seen that the electrodialysis has removed an alkaline constituent which is not essential to the physiological activity of insulin preparations. Lack of material made it impossible to determine the ash content of the other samples of sediment recorded in Table No. 8.

Attempts to remove the silica from the sediments were made by means of ultrafiltration through impermeable membranes and by re-electrodialysis with reversed polarity, thus drawing the sediment against gravity, but these were not successful. The per cent. of silica in the samples before and after these treatments was practically the same. The relatively low ash in sediment No. 16, with no decrease in physiological activity, indicates that the silica is probably not a part of the molecule of the active principle in insulin preparations.

A test for niotrogen, sulfur, and phosphorus by means of sodium fusions on active sediments Nos. 12, 13, and 16 was positive for the first two elements. No positive test for sulfur, phosphorus, or halogens could be obtained on the filtrate samples Nos. 12 and 16 (pH 4.8 material).

The nitrogen content of samples of sediment and filtrate was quantitatively determined by the micro-Kjeldahl technique of Pregl [22]. A sample weighing between 11 and 12 milligrams was used for each analysis. The weighed sample was dissolved in that volume of $^{N}/_{10}$ sulfuric acid which would give a final concentration of 1 milligram per cubic centimeter. Five cubic centimeters of this solution, measured by a calibrated

pipette, were used for each determination. This procedure made it possible to get 2 analyses on each sample using 5 milligrams of material for each determination. The results obtained from several analyses on sample of control, sediment and filtrate have been given in Table No. 8.

From this data it can be seen, that the percentage of Kjeldahl nitrogen in the sediments analyzed, based even on total solids, is fairly constant, while the nitrogen content for sediments Nos. 12, 13, and 16 based on total organic solids is constant within 0.96%, and averages 14.52% for the three samples.

The amphoteric nature of the active sediment, as shown by its ability to migrate either anodic or cathodic depending on the pH of the solution, and also by its solubility in both dilute acids and alkalies, when coupled with its precipitation by phosphotungstic acid, and its average nitrogen content of 14.52% makes it appear that the active principle of insulin either is a protein-like substance or is very closely linked to some protein. It is certain that the active material which deposits during the electrodialysis of insulin solutions, shows characteristic protein reactions, even though the active constituent in this sediment may not itself be a protein.

VIII. A Micro-crystalline Compound from the Active Sediment.

The appearance of the active sediment formed from insulin solutions subjected to electrodialysis, when examined under a microscope, indicated possibilities for crystallization. The relative insolubility of the active material in all general organic solvents rendered the usual crystallization technique inapplicable. Advantage was taken finally, of the solubility of the active sediment in acidified solutions.

Samples of active sediment were dissolved in dilute aqueous hydrochloric, acetic, and sulfuric acid solutions. These were permitted to evaporate slowly with the hope of obtaining the sediment in a crystalline condition. Several attempts were made but each time the material precipitated from the solution in an amorphous condition.

Another series of attempts were made using dilute sulfuric acid and ethyl alcohol as the solvent for the active sediment. Some of the solutions produced a micro-crystalline compound in very small quantity after standing for several days. The optimum condition for crystallization has not been established definitely, but equal volumes of 25% sulfuric acid and 95% ethyl alcohol produced crystals from two batches of active sediment in about 24 hours. This was the shortest crystallization time ever obtained, the usual time being at least one week.

The formation of the crystalline compound from the active sediment occurs in a very peculiar manner. The sediment, when first dissolved, gives a clear, pale yellow solution. The micro-crystals, in very small quantity, form in this acid solution after some of the alcohol has spontaneously evaporated. On longer standing, the crystals disappear, giving way to a turbid solution, from which an amorphous material precipitates. As more solvent evaporates this amorphous substance re-dissolves, giving a brownish colored solution from which, sometimes, on very long standing, the micro-crystalline substance is again formed. In several attempts, it has been impossible to get back the crystalline product even after standing for weeks. If a little ethyl alcohol is added to the liquid containing the amorphous substance after resolution of the crystals, the former re-dissolves, and in some cases, re-crystallization has been accomplished in this manner. However, if a little water is added to the liquid containing the amorphous substance, no resolution occurs, but even more amorphous material seems to come out of solution. If a large volume of water is added, complete resolution occurs but no crystals have ever been obtained from such diluted solutions.

The crystalline compound produced as described, has always had the same form, and has always shown the same relative insolubility in ordinary organic solvents and in water. The insolubility of the crystalline material in water, and the very small quantities in which it has been produced, together with the great difficulty experienced in attempting to free it from traces of mother liquor, have made the physiological and chemical investigation of the compound very difficult.

From the two microphotographs, plates Nos. 1 and 2, some idea may be had of the general appearance of the crystals under the microscope. Plate No. 1 shows a typical batch of crystals in contact with the mother liquor, while plate No. 2 shows a single group crystallizing in the usual radiating manner about some dust spot as a nucleus.

These crystals which are inactive are formed at the expense of the active sediment by the treatment described above. Their crystalline form and qualitative chemical reactions strongly suggest tyrosine. This aspect of the work opens up a new line of attack on the insulin problem.

Magnification 60×
Ordinary Light

Plate No. 1

Magnification 100×
Green Light

Plate No. 2

NOTE: The author wishes to thank Mr. E. P. Polushkin of the Department of Metallurgy for these micrographs.

DISCUSSION OF ERRORS

The greatest error in this investigation was that involved in the physiological assay of the activity in the various samples of material. This biological error is not due to faulty technique or inaccurate methods, but is due to the different reactions of the experimental animals to the insulin injection. In order to establish the validity of the conclusions drawn from the physiological tests, a series of at least three animals was run on each sample. The average error, calculated from the mean deviation for each series was found to be ± 16.19% for the blood sugar drop ¾ hour after injection, and ± 18.98% for the blood sugar drop 1¾ hours after injection. The error is larger for the second interval after injection because the results at this point are influenced to a greater degree by the individual reactions of the experimental animals. The error involved in weighing out the 2 milligram samples for the physiological tests becomes negligible in view of the much larger animal error.

The lack of material necessitated the use of colorimetric procedure for the hydrogen ion determinations. Since in this investigation, the colorimetric technique was open, not only to the usual optical and personal errors, but also to the so-called "Protein" error, no greater accuracy than a close approximation is claimed for the hydrogen ion determinations. The results are, however, sufficiently accurate to indicate the general trend of the hydrogen ion changes in the cases investigated.

The largest error involved in the ash determinations, is in the weighing of the samples (20 to 30 milligrams), and amounts to ± 0.33 to 0.50%.

The micro-Kjeldahl technique was perfected by analyses on chemically pure samples of acetanilide. The accuracy of the method can be seen from the following typical analyses.

Material Analyzed	Weight of sample	% N_2 Found by analysis	% N_2 Calculated from formula	% N_2 Difference from theory
c.p. acetanilide	6.6 mg.	10.53	10.37	+0.16
" "	7.4 mg.	10.70	10.37	+0.33
" "	7.4 mg.	10.82	10.37	+0.45
" "	7.2 mg.	10.82	10.37	+0.45
" "	7.1 mg.	10.70	10.37	+0.33

The standard sample weight in the actual analyses was 11 to 12 milligrams, bringing into the method a possible error of ± 0.83 to 0.91%.

All quantitative apparatus was calibrated and the errors involved were negligible in comparison to the errors discussed above.

SUMMARY

The results from (A) ultrafiltration and from (B) electrodialysis have led to the conclusions summarized under the respective headings.

A. Ultrafiltration.

1. The active principle of insulin can be ultrafiltered through collodion membranes of high and low permeability without loss of activity.
2. The nitrogen content of insulin is not changed by ultrafiltration through collodion membranes of high or low permeability.

B. Electrodialysis.

1. An active sediment can be deposited from insulin solutions when subjected to electrodialysis.
2. The sediment produced by the electrodialysis of insulin solutions appears to be 100% more active per unit of weight injected than the original material.
3. The filtrate, containing principally organic material, after removal of the active sediment, has little activity.
4. The electrode, at which the active sediment is deposited, is determined by the hydrogen ion concentration of the insulin solution undergoing electrodialysis.

5. There appears to be a definite hydrogen ion concentration at which the active sediment is produced from insulin solutions during electrodialysis.
6. The formation of the active sediment during the electrodialysis of insulin solutions, is not due to an isoelectric point precipitation.
7. The active sediment produced by the electrodialysis of insulin solutions shows protein reactions and contains organic sulphur.
8. A micro-crystalline compound can be obtained from the active sediment formed by the electrodialysis of insulin solutions by treatment with an alcohol, sulfuric acid and water mixture.

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VITA

Charles Ernest Braun was born in Brooklyn, N. Y., March 10, 1900. He attended the Brooklyn public schools, graduating from the Bushwick High School in 1918. He entered the Polytechnic Institute of Brooklyn in January, 1919, and was graduated with the degree of Bachelor of Science in Chemistry cum laude, in June, 1922. In September, 1922, he entered the Graduate School of Columbia University under the Faculty of Pure Science. After receiving the degree of Master of Arts in June, 1923, he continued the studies for the doctorate in the Faculty of Pure Science. From October, 1921 until June, 1922, he was assistant in general chemistry at the Polytechnic Institute of Brooklyn, and held the same position at Columbia University from October, 1923 until June, 1924, when he was appointed Assistant in organic chemistry. He is a member of the American Chemical Society, Phi Lambda Upsilon and Sigma Xi.

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